SYPHILIS

Molecular typing of *Treponema pallidum* strains from patients with neurosyphilis in Pretoria, South Africa

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Objective: To evaluate the molecular typing system for *Treponema pallidum* using cerebrospinal fluid (CSF) specimens obtained from patients with neurosyphilis in Pretoria, South Africa.
Methods: CSF specimens were collected from 32 men and 18 women with suspected late neurosyphilis. Typing of *T pallidum* involved PCR amplification and restriction analysis of the *tpr E*, *G* and *J* genes and determination of the number of 60 base pair tandem repeats within the *arp* gene by PCR amplification.
Results: Of 13 typeable specimens, 4 strain types were identified: 2*i*, 3*e*, 14*a* and 17*e*. Subtype 14*a* was identified in 7 specimens (53.8%), subtype 3*e* in 4 specimens (30.7%) and subtypes 17*e* and 2*i* in 1 specimen (7.6%) each.
Conclusions: This study shows that the typing system can be applied to specimens which may contain low numbers of spirochaetes such as CSF.

Although effective treatment has been available for more than six decades, syphilis remains a significant public health problem, especially in developing countries. The World Health Organization estimates that 12 million new cases of syphilis occur annually worldwide, of which approximately 4 million are in sub-Saharan Africa.[1] There has been a renewed interest in syphilis since ulcerative sexually transmitted infections (STIs) have been shown to be a risk factor for the transmission of HIV in HIV-infected individuals.[2–4] Similarly, the presence of ulcerative STIs in HIV-negative individuals may increase susceptibility to HIV infection by either disrupting the epithelial barrier or the presence of macrophages and CD4 T lymphocytes in the ulcer, which are target cells for HIV.[5 6] Syphilis is making a resurgence in many industrialised nations such as the US, Canada and the UK, where outbreaks either have occurred recently or are currently occurring.[7–9] The rise in incidence of neurosyphilis cases may be linked, in part, to individuals who are immunocompromised as a result of infection with HIV.[10–12]

Patients with ''early'' syphilis are defined as those with primary, secondary or early latent disease; patients with ''late'' syphilis are those with late latent disease, which includes late neurosyphilis.[13] Central nervous system invasion by *Treponema pallidum* may occur in up to 70% of individuals during early syphilis or in 15–20% of individuals during late neurosyphilis, which occurs years to decades after initial infection.[14 15] Although the host immune response should be taken into account with respect to disease progression after neuroinvasion, the manifestation of late neurosyphilis in some individuals may be associated with neurotropic strains of *T pallidum*. The biological plausibility for this hypothesis is supported by recent studies on *T pallidum* and *T pallidum*-related spirochaetes. For example, the neuroinvasive capacity of six *T pallidum* strains was recently studied in a rabbit model.[16] Using reverse transcriptase-PCR, *T pallidum* was detected more frequently in the cerebrospinal fluid (CSF) of rabbits infected with the Bal 73-1 and UW085B strains and, to a lesser extent, in Nichols, whereas CSF pleocytosis was not observed in these strains. On the other hand, CSF pleocytosis was observed in strains Sea 81-4 and Bal 7 but *T pallidum* DNA was detected only at one and two time points in the CSF of rabbits infected with these strains, respectively. Neither *T pallidum* DNA nor CSF pleocytosis was detected in rabbits infected with strain UW099B, suggesting that neuroinvasion did not occur with this strain. These data suggest that there are strain-specific differences in neuroinvasive capacity among the *T pallidum* strains studied. Studies on the three genospecies of *Borrelia burgdorferi* sensu lato, which cause lyme borreliosis, show that *B garinii* is the most frequently identified organism in the CSF of patients with neuroborreliosis in Europe, suggesting that this particular genospecies is more neuroinvasive than *B burgdorferi* sensu stricto and *B afzelii*.[17 18]

There is a paucity of data on the molecular epidemiology of syphilis, which is compounded by the fact that syphilis has different clinical manifestations and that *T pallidum* cannot be cultured on artificial laboratory media. Using a combination of PCR amplification and restriction fragment length polymorphism (RFLP) analysis of two different gene targets, a molecular typing system has been developed that can be used to differentiate between strains of *T pallidum*.[19] The typing system is based on the number of 60 base pair (bp) tandem repeats within the *arp* gene and a letter that denotes the RFLP profile of a segment of the *tpr E*, *G* and *J* genes. This method permits typing of strains by the amplification of *T pallidum* DNA directly from clinical specimens and circumvents the need to cultivate this organism in vitro. Previous typing studies have focused on genital ulcer specimens and to a lesser extent on blood for typing organisms.[19–23]

The objective of this study was to apply this typing system to CSF specimens, which often contain low numbers of spirochaetes, to examine the diversity of strains of *T pallidum* for patients diagnosed with late neurosyphilis.

Abbreviations: bp, base pair; CSF, cerebrospinal fluid; FTA-ABS, fluorescent treponemal antibody absorption; RFLP, restriction fragment length polymorphism; STI, sexually transmitted disease; VDRL, Venereal Disease Research Laboratory

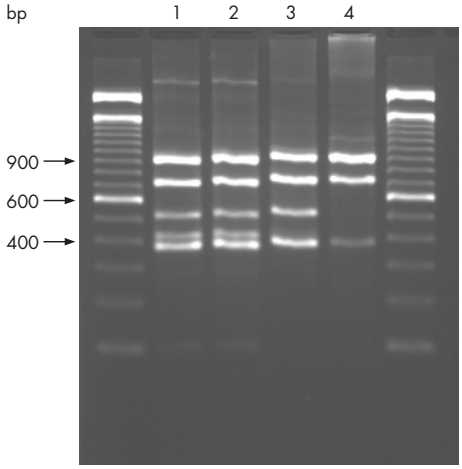

Figure 1 Representative samples with unique *Tru*9I restriction patterns. Lane 1, *Treponema pallidum* Nichols (restriction fragment length polymorphism (RFLP) pattern ''*a*''); lane 2, RFLP pattern ''*a*''; lane 3, RFLP pattern ''*e*''; lane 4, RFLP pattern ''*i*''.

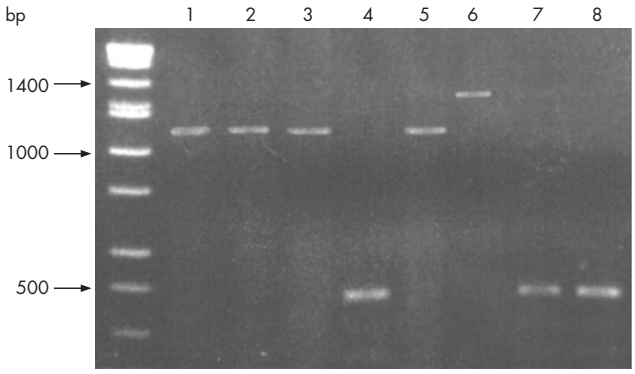

Figure 2 Representative samples with unique 60 base pair (bp) tandem repeat sizes within the *arp* gene. Lane 1, *Treponema pallidum* Nichols (1171 bp, 14 repeats); lanes 2, 3, and 5, 14 repeats; lanes 4, 7 and 8, 3 repeats (510 bp); lane 6, 17 repeats (1350 bp).

METHODS

Between June 1999 and September 2000, a total of 50 CSF specimens were collected from adult patients (32 men and 18 women) with suspected neurosyphilis. This was based on differential diagnosis for a variety of clinical presentations and the patients were admitted to the neurology wards at the Dr George Mukhari Hospital, GaRankuwa, Pretoria, South Africa. The clinical stage of disease suspected was tertiary syphilis in all patients. All CSF specimens were assayed immediately after collection using Venereal Disease Research Laboratory (VDRL; Behring Diagnostics, Marburg, Germany) and fluorescent treponemal antibody absorption (FTA-ABS) IgG (Diagnostic and Technical Services, Randburg, South Africa) tests as per the manufacturer's instructions. Patients were diagnosed as having neurosyphilis if their CSF specimens were positive according to the FTA-ABS IgG and VDRL tests. The HIV status of the patients was unknown at the time the specimens were collected.

A CSF sample that previously tested positive was used as a positive control for the VDRL test and normal saline served as a negative control. A reactive control serum with human *T pallidum* antibody and a non-specific human control serum were used as positive and negative controls for the FTA-ABS test, respectively. CSF specimens were stored at -70℃ before molecular testing. This study was approved by the Faculty of Medicine's Research, Ethics and Publications Committee of the University of Limpopo, Pretoria, South Africa.

Genomic DNA was extracted from a 100 µl aliquot of CSF using the Gene-Releaser method (Bioventures, Murfreesboro, Tennessee, USA) according to the manufacturer's instructions. CSF specimens were heated for 10 min in a water bath at 100℃ and then centrifuged for 1 min at 12 000 rpm. The supernatants were added to filter tubes (Whatman International, Ann Arbor, Michigan, USA) and centrifuged for 20 min at 5000 rpm to concentrate the DNA. Gene-Releaser beads (20 µl) were added to each filter and, after mixing, the DNA–bead mixture was transferred to a 0.2 ml PCR tube and the Gene Releaser DNA extraction protocol was performed in a MiniCycler PTC-150 thermocycler (MJ Research, Waltham, Massachusetts, USA) as follows: 65℃ for 0.5 min, 8℃ for 0.5 min, 65℃ for 1.5 min, 97℃ for 3 min, 8℃ for 3 min, 65℃ for 3 min, 97℃ for 1 min and 65℃ for 1 min. The final step consisted of a hold at 80℃ for 25 min. After DNA extraction, the reaction mixtures were centrifuged at 12 000 rpm for 1 min and the supernatants were aspirated and used as templates in PCR assays. DNA samples were stored at –20℃ until tested.

A diagnostic PCR assay with primers K03A (5′-GAAGTTTG TCCCAGTTGCGGTT-3′) and K04A (5′-AGAGCCATCAGCCCTT TTCA-3′) was used to amplify a 261 bp fragment of the 47 kDa protein gene of *T pallidum*.[24] The PCR mixture contained 10 µl of DNA template, 10 µl of 10× PCR buffer (100 mM Tris–HCl, 500 mM KCl, 1% Triton X-100; Promega, Madison, Wisconsin, USA), 4 µl deoxynucleoside triphosphates (10 mM each of dTTP, dCTP, dATP and dGTP, Promega), 25 mM $MgCl_2$ (Promega), 12.5 pmol of each primer and 0.5 U *Taq* polymerase (Promega) in a 100 µl reaction volume. PCR amplification was performed as follows: 40 cycles of 95℃ for 1 min, 61℃ for 1 min and 72℃ for 1 min. Final extension consisted of one cycle at 72℃ for 10 min.

Typing experiments were basically performed as described previously, but with a few modifications.[19 20] PCR amplification of the 60 bp tandem repeat region within the *arp* gene was performed in a total reaction volume of 50 µl. The PCR mixture contained 10 µl of DNA template, 5 µl of 10× expand high fidelity reaction buffer (20 mM Tris-HCl, 100 mM KCl, 1 mM Dithiothreitol, 0.1 mM EDTA, 0.5% Tween 20, 50% glycerol, 0.5% Nonidet P40; Roche Diagnostics, Mannheim, Germany), 1 µl deoxynucleoside triphosphates (10 mM each of dTTP, dCTP, dATP and dGTP), 25 mM $MgCl_2$ (Promega), 100 pmol of each primer and 0.87 U expand high fidelity *Taq* polymerase (Roche Diagnostics). The primer pair consisted of the 20-mer forward primer ARP1 (5′-CAAGTCAGGACGGACTGTCC-3′) and the 18-mer reverse primer ARP2 (5′-GGTATCACCTGGGG ATGC-3′). PCR amplification consisted of an initial denaturation step at 94℃ for 4 min, followed by 45 cycles at 94℃ for 1 min, 60℃ for 1 min and 68℃ for 5 min. The final extension consisted of one cycle at 68℃ for 15 min. PCR products were resolved by electrophoresis on a 2% agarose gel at 100 V for 1 h, together with a 100 bp DNA ladder (Life Technologies, Paisley, UK). The number of *arp* gene tandem repeats was estimated by comparison with the molecular weight marker fragments and the *arp* amplicon from the Nichols strain of *T pallidum* (14 repeats).

A two-step nested PCR was performed to amplify the *tpr E*, *G* and *J* genes. Oligonucleotides for the first PCR consisted of the 20-mer forward primer B1 (5′-ACTGGCTCTGCCACACTTGA-3′) and the 20-mer reverse primer A2 (5′-CTACCAGGAGAGGG TGACGC-3′), which amplify a 2186 bp region of the *tpr E*, *G* and *J* genes. The PCR mixture contained 10 µl of DNA template, 10 µl of 10× expand high fidelity reaction buffer containing 10 mM $MgCl_2$ (Roche Diagnostics), 2 µl dNTPs

Table 1 Results of serological and diagnostic PCR assays (n = 50)

		Cerebrospinal fluid serology		
		VDRL/FTA-ABS IgG + (n = 35)	VDRL/FTA-ABS IgG − (n = 10)	VDRL negative, FTA-ABS IgG + (n = 5)
PCR	+	23	5	0
	−	12	5	5

FTA-ABS, fluorescent treponemal antibody absorption; VDRL, Venereal Disease Research Laboratory; +, positive; −, negative.

(10 mM each of dTTP, dCTP, dATP and dGTP; Roche Diagnostics), 60 pmol of each primer and 2.6 U expand high fidelity *Taq* polymerase (Roche Diagnostics) in a reaction volume of 100 µl. PCR amplification was performed as follows: one cycle at 94℃ for 5 min, followed by 45 cycles at 94℃ for 1 min, 60℃ for 2 min and 68℃ for 2.5 min. The final extension consisted of one cycle at 68℃ for 15 min. Subsequently, a nested PCR assay was performed, which amplified an approximately 1836 bp internal region of the 2186 bp PCR product. The PCR primers used were the 18-mer forward primer IP6 (5′-CAGGTTTTGCCGTTAAGC-3′) and the 20-mer reverse primer IP7 (5′-AATCAAGGGAGAATACCGTC-3′). The PCR mixture contained 10 µl DNA template from the first *tpr* PCR, 10 µl of 10× expand high fidelity reaction buffer containing 10 mM $MgCl_2$ (Roche Diagnostics), 2 µl dNTPs (10 mM each of dTTP, dCTP, dATP and dGTP), 60 pmol of each primer and 2.6 U expand high fidelity *Taq* polymerase in a total volume of 100 µl. PCR amplification was performed as follows: one cycle at 94℃ for 5 min, followed by 40 cycles at 94℃ for 1 min, 59℃ for 1 min and 68℃ for 2 min. The final extension consisted of one cycle at 68℃ for 15 min.

Unpurified amplicons from the nested PCR assay were digested with the restriction enzyme *Tru*9I (Roche Diagnostics), which is an isoschizomer of *Mse*I. The restriction mixture contained 10 µl of PCR amplicon, 2 µl SureCut buffer (10 mM Tris–HCl, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM Dithiothreitol) and 10 U restriction enzyme *Tru*9I. The reaction mixture was incubated overnight at 37℃. Restriction fragments were electrophoresed as for *arp* amplicons. Agarose gels were stained with a 0.5 µg/ml ethidium bromide (Sigma-Aldrich, Paisley, UK) solution and visualised with an ultraviolet transilluminator. Restriction fragments sizes were estimated by comparison with the 100 bp molecular weight marker fragments and the *Tru*9I restriction fragments of the *tpr* amplicon of the Nichols strain. All PCR amplifications were performed in an OmniGene thermocycler model TR3 CM220 (Hybaid, Ashford, UK).

Stringent laboratory procedures were implemented to avoid contamination of CSF specimens or samples, including a dedicated room with a laminar flow hood for DNA extraction, use of aerosol-free pipette tips and separate pipettes for DNA extraction and PCR set-up. For all PCR assays, a positive and a no-template control were included in each run.

RESULTS

In all, 35 of 50 (70%) CSF specimens were VDRL and FTA-ABS IgG positive (table 1), 10 (20%) gave negative results in both assays and the remaining 5 (10%) were VDRL-negative FTA-ABS IgG positive.

Of the 50 CSF specimens tested, 28 (56%) were positive in the diagnostic PCR targeting the 47 kDa gene of *T pallidum* (table 1). In all, 15 of the 28 positive specimens had sufficient material for typing, of which 13 (87%) were typeable; 2 specimens gave partial results. Of the 13 specimens, 12 were VDRL and FTA-ABS IgG positive and 1 specimen was VDRL and FTA-ABS IgG negative. Four specimens that were VDRL and FTA-ABS IgG negative were positive with the diagnostic PCR but negative with the typing PCR assays. Figures 1 and 2 show the unique *Tru*9I RFLP patterns of the *tpr E, G* and *J* genes and representative samples of different *arp* repeat sizes, respectively.

By combining the number of 60 bp repeats of the *arp* gene and the *Tru*9I RFLP pattern of the *tpr E*, *G*, and *J* genes, four strain types (2*i*, 3*e*, 14*a*, 17*e*) were identified among the 13 typeable specimens. The most common subtype, 14*a*, was found in 7 of 13 specimens (53.8%), followed by subtype 3*e*, found in 4 of 13 specimens (30.7%); 1 specimen each (7.6%) had subtype 17*e* and 2*i*.

DISCUSSION

The previously described typing system, which is based on the number of 60 bp repeats within the *arp* gene and a lower-case letter which denotes the *Mse*I (*Tru* 9I) RFLP profile of the *tpr E*, *G*, and *J* genes, was used in this study to type *T pallidum* DNA in CSF specimens from patients with late neurosyphilis.[19 20] Using this typing system, four different strain types (2*i*, 3*e*, 14*a* and 17*e*) were identified among 13 typeable specimens; the 2*i*, 3*e* and 17*e* strain types were not found in previous studies.[19–22] The *e* and *i* RFLP patterns of the *tpr* genes have been described previously; however, strains with only two and three 60 bp repeats within the *arp* gene are unique to this study.[19–21]

The two specimens that gave partial typing results were positive by the *tpr* PCR assay only and had RFLP patterns *a* and *e*. The inability to obtain an amplicon with the *arp* PCR assay could have been due to these being early manifestations of late neurosyphilis infections as suggested by the low VDRL titres (1:2), or to a large number of 60 bp repeats (>17) within the *arp* gene, resulting in an undetectable amplicon on an agarose gel. Four of five specimens that were VDRL and FTA-ABS IgG negative and diagnostic PCR positive could not be typed, suggesting that these were from patients with early manifestations of late neurosyphilis infections and low number of spirochaetes. It is unlikely that diagnostic PCR results were due to cross-contamination during PCR set-up because a no template control included in each run gave the expected results.

Interestingly, we did not find any *T pallidum* strains with subtype 14*d*, although this subtype has been commonly found in genital ulcer specimens throughout South Africa during the same time period.[20] The identification of four different *T pallidum* strain types in this study suggests that a single strain type is not uniquely associated with neurosyphilis. However, some individuals may have been HIV positive, which could have accounted for the wider variety of strains observed because neuroinvasion in these individuals may depend more on their immune status than on characteristics of the infecting strain. The observation that the majority of specimens (54%) had the 14*a* strain type is intriguing, considering that the Nichols strain of *T pallidum*, which has the same type, was originally isolated from the CSF of a patient with secondary syphilis.[25] In addition, the 14*a* strain type may have been circulating in areas where these patients acquired syphilis and because the length of time between acquisition of primary syphilis and the manifestation of signs and symptoms of neurosyphilis is unknown, the possible association of this strain type with neurosyphilis, or lack thereof, cannot be confirmed.

The typing system described by Pillay *et al*[19 20] is robust and can be successfully applied to CSF specimens, which usually contain low numbers of spirochaetes. This broadens the applicability of the typing system to include specimens obtained from patients with primary, secondary or tertiary syphilis. In addition, because typing assays were performed independently at the University of Limpopo, South Africa, it demonstrates that the methodology can be successfully applied in different

Key messages

- This study describes the successful application of the *Treponema pallidum* typing system to cerebrospinal fluid (CSF) specimens obtained from patients diagnosed with late neurosyphilis.
- The majority of *T pallidum* strains (54%) had the 14a strain pattern, which suggests that this strain may be more neuroinvasive than other strains.
- Epidemiological studies are needed to identify potentially neurotropic strains by comparing strain types from patients with primary syphilis and those from patients with neurosyphilis.
- Patients with positive CSF VDRLs are likely to have sufficient treponemes in their CSF to provide enough DNA for typing.

laboratories. However, to prove that the typing results can be reproduced, it will be necessary to type the same specimens in different laboratories. Further studies are needed to determine whether specific *T pallidum* strain types are associated with the different clinical manifestations of syphilis.

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